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Semaphorin 7A Is a Negative Regulator of T Cell Responses

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Summary

Semaphorins play an essential role in axonal guidance, and emerging evidence points to diverse functions of several Semaphorin family members in the immune system. Semaphorin 7A (Sema7A) promotes axonal growth in the central nervous system. Here, we show that Sema7A also plays a critical role in negative regulation of T cell activation and function. T cells deficient in Sema7A exhibit enhanced homeostatic and antigen-induced proliferative response. Moreover, autoreactive Sema7A-deficient T cells mediate aggressive autoimmune disease. The deficiency in Sema7A leads to defective TCR downmodulation and T cell hyperresponsiveness. These results demonstrate an important role of Sema7A in limiting autoimmune responses and add to growing evidence of shared signaling pathways used by the immune and nervous systems.

Introduction

Semaphorins are neuronal guidance factors that were initially characterized by their ability to inhibit axonal migration via chemorepulsive mechanisms, which induce cytoskeletal rearrangement and growth cone collapse (Kolodkin et al., 1992, 1993; Luo et al., 1993). Semaphorins transduce the repulsive signal through receptors of the plexin family (Tamagnone et al., 1999), and in addition, two of the class IV semaphorins interact with receptors structurally unrelated to plexins (Kumanogoh et al., 2000, 2002b).

Several members of the semaphorin family have been characterized with respect to their function in immunity. Sema4D is a 150 kDa transmembrane protein, which belongs to the class IV semaphorin subfamily (Delaire et al., 1998). In lymphoid organs, Sema4D is abundant on resting T cells. The receptor for Sema4D in lymphoid tissues is CD72 (Elhabazi et al., 2003; Kumanogoh et al., 2000). CD72 functions as a negative regulator of B cell responses, and Sema4D binds CD72 and turns off its inhibitory signaling during T cell-mediated B cell activation (Kumanogoh and Kikutani, 2001). Another immune semaphorin, Sema4A is expressed by DCs, B cells, and by activated T cells. Sema4A costimulates T cell activation by interaction with receptor Tim-2 (Kumanogoh et al., 2002a). Studies of Sema4A-deficient mice show that DC-derived Sema4A is important for T cell priming, while T cell-derived Sema4A is involved in developing Th1 responses (Kumanogoh et al., 2005).

Semaphorin 7A, the only GPI-linked protein in semaphorin family, is prominently expressed in the embryo and in the lymphoid organs and the nervous system of adult mice (Sato and Takahashi, 1998). Sema7A is a cellular homolog of viral semaphorins encoded by vaccinia and herpesvirus (Comeau et al., 1998; Lange et al., 1998; Xu et al., 1998) and was demonstrated to bind to the cellular receptor Plexin C1 in vitro (Tamagnone et al., 1999). Sema7A can induce monocyte chemotaxis and cytokine production and is expressed in activated lymphocytes and thymocytes (Holmes et al., 2002; Mine et al., 2000), suggesting an immune function for this molecule. In addition, Sema7A is highly expressed on most human T lymphocytes and natural killer cells (Angelisova et al., 1999). Sema7A knockout animals exhibited diminished axonal tracts formation, while treatment with soluble Sema7A enhanced axonal outgrowth (Pasterkamp et al., 2003). Sema7A binding to neurons induced activation of focal adhesion kinase (FAK) and extracellular regulated kinases (ERKs) (Pasterkamp et al., 2003). These effects of Sema7A were mediated by β1-integrin and were independent of Plexin C1 (Pasterkamp et al., 2003). Thus, Sema7A binds to at least two receptors with very different activities, and the significance of these interactions in various tissue types remains to be clarified.

The expression of Sema7A on T lymphocytes and the acquisition of its homolog by two viral families suggest that this molecule may have an important specific function in lymphocytes. Here, we investigate the role of Sema7A in T cells and demonstrate that it plays a T cell-intrinsic inhibitory role and is essential for limiting T cell-mediated autoimmunity.

Results

Sema7A-Deficient T Cells Are Hyperresponsive

Sema7A is expressed in naive CD4 and CD8 T cells, and its expression is increased in activated CD4 T cells and remains elevated in differentiated T helper 1 (Th1) lymphocytes (Figure S1A). Sema7A mRNA is detected in dendritic cells (DCs) and macrophages but is not prominently expressed in B cells (Figures S1A and S1B). To address the function of Sema7A in the immune system, we first compared T cell responses in Sema7A$^{-/-}$ animals and their wt littermates. Mice were immunized with a model protein antigen, chicken ovalbumin (OVA) emulsified in adjuvant. Six days after immunization, CD4 T cells were purified from the draining lymph nodes and restimulated with wt antigen-presenting cells (APC) pulsed with different doses of OVA. Sema7A-deficient

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T cells showed increased proliferative response to the antigen, compared to the wt T cells (Figure 1A), suggesting that Sema7A may have a negative regulatory role in T cell activation. Sema7A deficiency leading to increased proliferation could reside either in antigen-presenting cells or in T cells. To distinguish between these possibilities, we performed in vitro T cell activation assays with naive OTII TCR transgenic CD4 T cells stimulated with cognate peptide presented by wt APCs. OTII CD4 T cells on Sema7A$^{-/-}$ background showed dramatically increased proliferative response to cognate antigen presented by wt APC, compared to OTII Sema7A$^{+/+}$ T cells (Figure 1B). This result demonstrated that the increased T cell responsiveness was due to the lack of Sema7A in T cells and suggested that this molecule may have an inhibitory function that regulates proliferation of T cells. In order to further study the inhibitory role of Sema7A in T cell activation, we prepared recombinant soluble Sema7A-Fc. Addition of 100 μg/ml of soluble Sema7A-Fc to wt OTII transgenic T cells stimulated with OVA-pulsed DCs resulted in a significant decrease in T cell proliferation, compared to addition of Fc alone (Figure 1C). This result provides further indication for an inhibitory function of Sema7A in T cell activation.

We next addressed the role of Sema7A expression in DCs by comparing the maturation and T cell activation by wt and Sema7A$^{-/-}$ DCs. No significant differences were observed in DC maturation induced by various TLR ligands in vitro (Figure S2A). Wt and Sema7A$^{-/-}$ DCs had equivalent ability to activate naive T lymphocytes in vitro (Figure S2B) and in vivo (Figure S2C). These findings indicate that the increased proliferative response seen in Sema7A$^{-/-}$ T cells is likely to be T cell autonomous and independent of Sema7A deficiency in APCs.

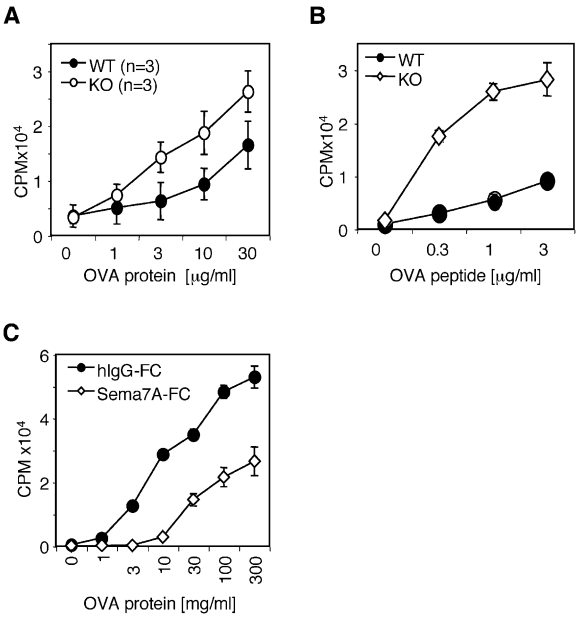


Figure 1. Function of Sema7A in T Cell Proliferation

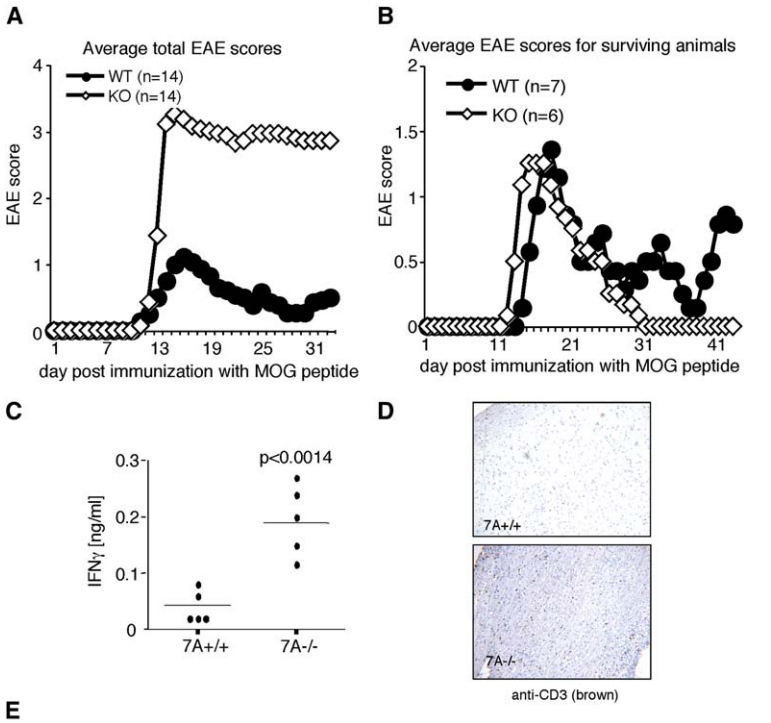
(A and B) Enhanced CD4 T cell responses in Sema7A-deficient mice compared with wild-type mice. (A) CD4 T cell responses from OVA immunized mice. (B) Proliferation of naive OTII CD4 Sema7A$^{-/-}$ or Sema7A$^{+/+}$ T cells activated with wild-type APCs.

(C) Addition of soluble, recombinant Sema7A-FC (100 μg/ml) inhibits proliferation of naive OTII CD4 T cells stimulated with Ovalbumin-pulsed DCs. As a control, recombinant IgG-FC fragment was added at 100 μg/ml. T cell-proliferative responses are expressed as the average ± SE of three animals per group in (A) or as the mean ± SE of triplicate cultures in (B) and (C).

Severe EAE in Sema7A-Deficient Mice

To examine the in vivo function of Sema7A in CD4 T cells, we employed the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), which is an inflammatory disease of the central nervous system (CNS) mediated primarily by Th1 cells (Kuchroo et al., 2002; Wong et al., 1999). Upon induction of EAE, self-reactive activated T cells transmigrate into the CNS, where they activate resident microglia and attract further influx of monocytes and other cells through the secretion of proinflammatory cytokines (Raine, 1997; Ruddle et al., 1990). To analyze the role of Sema7A in T cell responses in vivo, we immunized Sema7A$^{-/-}$ and wt littermate mice with myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide in complete Freud's adjuvant (CFA). This immunization regimen leads to the induction of EAE within 12–15 days (see Experimental Procedures). Comparison of mean disease scores (averaged from three independent experiments) showed a dramatically increased disease index in Sema7A$^{-/-}$ mice (Figures 2A and 2E), which was due to the high mortality (~60%) (Figure 2E) of these animals near the time of disease onset. Analysis of disease progression in surviving Sema7A$^{-/-}$ mice showed a single, lingering paralysis episode in the Sema7A-deficient animals, in contrast to a later onset, earlier disease remittance, and typical consecutive episodes of relapse in the wt littermates (Figures 2B and 2E). Serum levels of IFNγ measured on day 10 postimmunization were elevated (Figure 2C), and, consistently, a larger number of infiltrating lymphocytes was found in the spinal cord sections from moribund Sema7A$^{-/-}$ animals compared to wt animals on the same day postimmunization (Figure 2D). Additionally, the level of IL-12 in the serum of Sema7A$^{-/-}$ mice was also found to be elevated (Figure S3A). As the aggravated autoimmunity could result from a deficiency in cytokines that play a regulatory role in inflammation, we measured the levels of IL-10 and TGF-β in the serum of animals prior to and post EAE immunization but found no significant differences in their levels between the Sema7A$^{+/+}$ and Sema7A$^{-/-}$ littermates (Figures S3B and S3C). Therefore, the high mortality of Sema7A$^{-/-}$ animals at the onset of EAE is likely a result of increased T cell activation, accelerated infiltration of T cells into the CNS, and the elevated levels of IFNγ produced by activated T cells. This phenotype is indicative of an exacerbated autoimmune response in Sema7A$^{-/-}$ animals.

The CD25 CD4 regulatory T cells play an important role in autoimmune disease protection (Paust and Cantor, 2005; Sakaguchi and Sakaguchi, 2005), and their deficiency could contribute to the severity of EAE observed in Sema7A$^{-/-}$ animals. We therefore addressed the in vivo functionality of regulatory T cells deficient in Sema7A by using a T cell transfer model of colitis. In this model, intraperitoneal injection of a small number of purified CD4 RB^{high} CD25^{negative} T cells into a lymphopenic host results in colitis and is associated with a rapid weight loss in the



E

Summary of the EAE disease course in Sema7A+/+ vs Sema7A-/- mice

Mouse Strain	No. of mice	Incidence	%Mortality	Average Day of onset	Average Cumulative Disease score
Sema7A+/+	14	92 (13/14)	0 (0/14)	14.9	0.39
Sema7A-/-	14	100 (14/14)	61 (8/14)	12.1	1.86

Figure 2. Increased Morbidity and Alteration in the Clinical Course of EAE in Sema7A$^{-/-}$ Mice

EAE was induced and scored in Sema7A$^{+/+}$ (n = 14) and Sema7A$^{-/-}$ (n = 14) mice with MOG_{35-55}. Pooled data from three independent experiments are shown.

(A) Sema7A$^{-/-}$ mice are prone to die at the onset of EAE.

(B) Sema7A$^{-/-}$ animals surviving past the disease onset exhibit a monophasic EAE with a delayed period of recovery, compared to relapsing-remitting EAE phenotype in wild-type animals.

(C) Elevated levels of IFNγ in the serum of Sema7A$^{-/-}$ mice 11 days postimmunization ($p < 0.0014$, result of unpaired t test with one-tailed p value).

(D) Increased infiltration of CD3 lymphocytes into CNS in moribund Sema7A$^{-/-}$ animals 11 days postimmunization with MOG.

(E) Summary of EAE course in Sema7A$^{-/-}$ and Sema7A$^{+/+}$ mice. Sema7A$^{-/-}$ mice are prone to increased mortality (61%) at the onset of disease compared to Sema7A$^{+/+}$ (0%) littermates. The onset of disease in Sema7A$^{-/-}$ mice is accelerated (mean 12.1 days compared with 14.9 days in Sema7A$^{+/+}$).

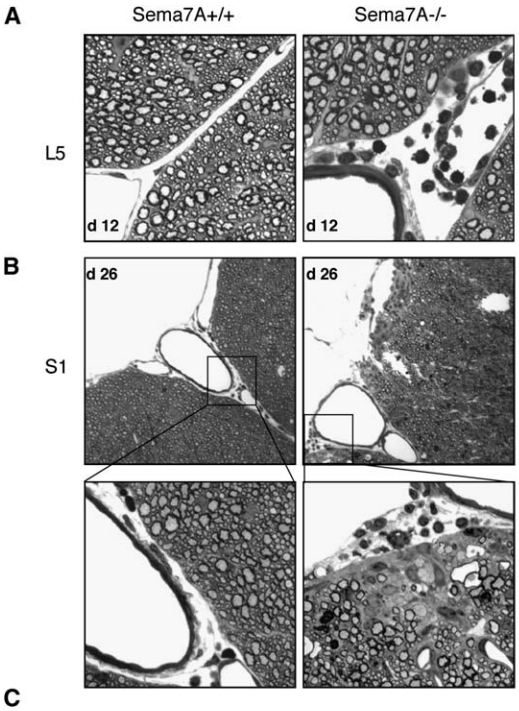
animal (Powrie et al., 1994). Rag2$^{-/-}$ host mice transferred with Sema7A$^{-/-}$ or wt CD4 RB^{high} CD25^{negative} T cells developed comparable weight loss and clinical signs of colitis (Figure S4A and data not shown). Cotransfer of Sema7A$^{-/-}$ or Sema7A$^{+/+}$ CD25^{high} CD4 T regulatory cells equally suppressed the colitis-associated weight loss and clinical signs of the disease (Figure S4B and data not shown). This result suggests that Sema7A$^{-/-}$ regulatory T cells are functional and further points to a specific defect in the naive CD4 T cells and to their abnormal activation as a cause of the aggravated EAE in Sema7A$^{-/-}$ animals.

Exacerbated EAE Pathology in Sema7A-Deficient Mice

Sema7A is a neuronal factor highly expressed in the brain, where it could potentially contribute to neuronal protection during an autoimmune disease. In order to evaluate if Sema7A deficiency makes neurons more vulnerable to damage during EAE, we examined histopathological changes in the CNS of wt and KO animals with similar clinical scores. Despite the similarity in clinical scores between the two groups of surviving animals (Figure 2B), there were differences at the level of histopathology of the lower lumbar region of the spinal cord. In Sema7A$^{+/+}$ mice in the early phase (12 days postimmunization, prior to EAE onset), very little inflammation was detectable (Figure 3A, left panel). In contrast, in Sema7A$^{-/-}$ mice, there was a noticeable influx of mononuclear cells to the spinal cord at the same time points (Figure 3A, right panel). At day 26 postimmunization, wt and KO animals of equal clinical scores (EAE = 0.5) were compared. Inflammation and demyelination were more pronounced in the lower spinal cord of Sema7A$^{-/-}$ mice compared to wt animals, with Wallerian degeneration, and remyelination (Figure 3B, lower right panel). However, while inflammation and demyelination were more widespread and appeared earlier in Sema7A$^{-/-}$ mice (Figure 3C), no additional neuronal changes unrelated to inflammation were observed. Therefore, Sema7A deficiency in the EAE model appears to affect only cells of the immune system and does not seem to play any detectable role in neuronal protection.

Enhanced Delayed Type Hypersensitivity Response in Sema7A$^{-/-}$ Mice

To further determine if the lack of Sema7A is responsible for a generalized defect in activation of CD4 T cells in vivo, we analyzed the induction of a delayed type hypersensitivity (DTH) response by sensitizing Sema7A$^{-/-}$ and wt animals with a chemical skin irritant, oxazolone. Activated CD4 T cells play a major role in DTH by emigrating to the irritated skin areas and producing inflammatory cytokines, resulting in a local tissue swelling (Kobayashi et al., 2001). The extent of ear swelling, measured 48 hr postchallenge, was significantly greater in the Sema7A$^{-/-}$ than in wt animals upon secondary challenge (Figure S5). This exaggerated DTH response



C

Lower spinal cord (L5, L6, S1)

	Cell infiltrates		Demyelination	
	7A+/+	7A-/-	7A+/+	7A-/-
12 days p.i.	0.25	0.75	0	0
26 days p.i.	0.5	1.0	0.5	0.75

Figure 3. Histopathology of EAE in Sema7A$^{+/+}$ and Sema7A$^{-/-}$ Mice

(A and B) Toluidine-blue-stained epoxy sections (1 μm) from the lower lumbar spinal cord region. (A) At 12 days postinduction of EAE, Sema7A$^{-/-}$ mice show inflammation and increased number of lymphocytes in L5 region of spinal cord, which is not seen in wt littermates. (B) 26 days postimmunization, sections from Sema7A$^{-/-}$ mice (B, right) (S1 spinal cord) showed infiltration of lymphocytes, and demyelination (lower right). Demyelination was less pronounced in Sema7A$^{+/+}$ mice (B, left) (S1 spinal cord), but an influx of inflammatory cells was observed.

(C) Distribution and severity of histopathological signs. Sections of representative mice were evaluated blindly with scores between 0.5–4 for inflammatory cell infiltrates and demyelinated axons. Means are based on three slides with two animals per group.

is consistent with the hyperactive T cell phenotype seen in EAE.

T Cell-Autonomous Role of Sema7A in Limiting Autoimmunity

While the pathology of EAE is mediated in large part by T cells, the contribution of other cells types is essential (Kuchroo et al., 2002) and could potentially play a role in the high mortality of Sema7A$^{-/-}$ mice at the onset of EAE. To determine the contribution of Sema7A deficiency specifically in T cells, we purified naive CD4 T cells from Sema7A$^{-/-}$ or wt littermates, labeled them with CFSE, and transferred one million cells per mouse into Sema7A$^{+/+}$, T cell-deficient recipients (Rag2$^{-/-}$ or TCR$\alpha^{-/-}$). Twenty-four hours later, mice were immunized with MOG$_{35-55}$. Within 6–9 days, all the mice receiving Sema7A$^{-/-}$ T cell, but none of the Sema7A$^{+/+}$ T cell recipients, became moribund or died (Figure 4A). Sema7A$^{-/-}$ T cell recipients had higher serum concentrations of IFNγ, IL-6, and KC-1, compared to Sema7A$^{+/+}$ T cell-recipient mice (Figure S3D and data not shown). The immunofluorescent staining revealed higher numbers of Sema7A$^{-/-}$ CD4 T cells in the cerebellar sections from transfer recipients, which were not seen in wt T cell recipients (Figure 4B, left panels). The bright CD11b staining, indicative of activated microglia, is a likely effect of IFNγ production by infiltrating Sema7A$^{-/-}$ CD4 T cells and was not observed in wt T cell recipients (Figure 4B, right panels). The activated Sema7A$^{-/-}$ T cells displayed a robust expansion, making up to 50% of total cells in the spleen, compared to a more modest proliferation (2%–3% of total spleen cells) in wt T cell recipients (Figures 4C and 4D). Sema7A$^{-/-}$ CD4 T cells showed increased expression of activation markers, such as CD25, compared to Sema7A$^{+/+}$ T cells (Figure 4E). Thus, the hyperactivity of Sema7A$^{-/-}$ T cells is likely due to the lack of a T cell-autonomous inhibitory effect of Sema7A, as all other cells in the recipient mice were Sema7A sufficient.

Increased Homeostatic Proliferation of Sema7A-Deficient T Cells

We next examined the role of Sema7A in T cells in homeostatic proliferation. Homeostatic proliferation in lymphopenic hosts is controlled primarily by cytokines and low-affinity interactions of T cells with self-antigens and has been linked with T cell-mediated autoimmunity (Gallegos and Bevan, 2004; Surh and Sprent, 2000). We transferred one million of naive Sema7A$^{-/-}$ or littermate wt CD4 T cells into Rag2$^{-/-}$;Sema7A$^{+/+}$ lymphopenic hosts to compare their homeostatic proliferation. Within 6 days posttransfer, the Sema7A$^{-/-}$ T cells showed greater expansion compared to wt T cells (Figures 5A and 5B). Time-course analysis revealed that Sema7A$^{-/-}$ CD4 T cells showed a greater degree and faster kinetics of proliferation compared to Sema7A$^{+/+}$ T cells (Figure 5C). These data demonstrate that Sema7A expressed in T cells negatively regulates lymphopenia-induced T cell proliferation.

Sema7A Negatively Regulates TCR Downmodulation and Signaling

TCR-mediated T cell activation is controlled by a number of parameters, including the duration of the TCR signaling. Upon TCR stimulation, cell surface expression of TCR declines rapidly as components of the TCR signaling complex are targeted for degradation through the endocytic pathway (Duan et al., 2004; Geisler, 2004). Sustained surface expression of TCR can interfere with termination of downstream signals and result in prolonged triggering of the T cells, causing their hyperactivation (Naramura et al., 2002). To determine the effect of Sema7A on TCR signaling, we activated T cells with plate-bound anti-CD3 antibody and analyzed changes in the TCR surface expression by flow cytometry. We found that anti-CD3 stimulation led to a downregulation of surface TCR expression in Sema7A$^{+/+}$ CD4 T cells, whereas this TCR downregulation was markedly delayed in Sema7A$^{-/-}$ T cells (Figure 6A). After 8 hr of anti-CD3 stimulation, the wt cells lost about 90% of their TCR surface expression. In contrast, the Sema7A$^{-/-}$ CD4 T cells had retained more than 40% of the TCR level

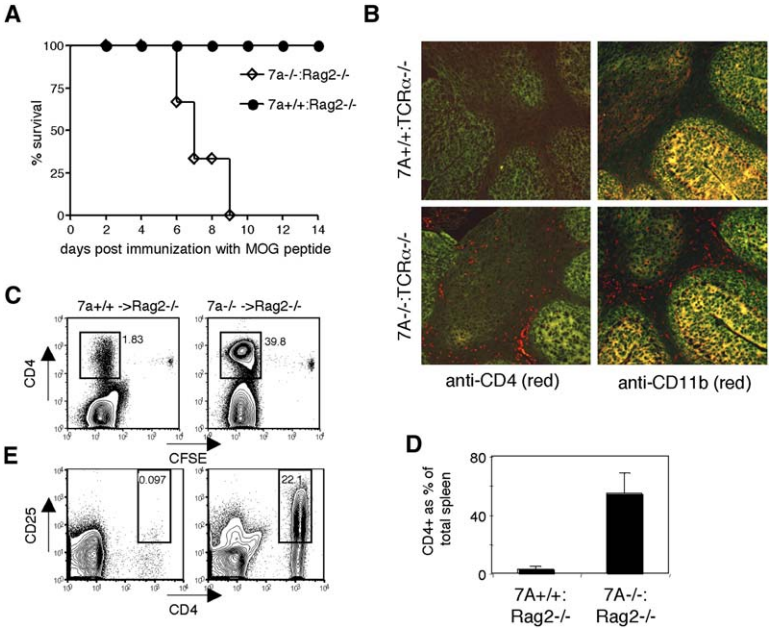


Figure 4. Increased Expansion of Sema7A$^{-/-}$ CD4 T Cells in a Lymphopenic Host

Rag2$^{-/-}$ mice received 1 × 10^6 FACS-sorted CFSE labeled CD4 cells from Sema7A$^{-/-}$ or Sema7A$^{+/+}$ littermates intravenously. 1 day posttransfer, mice were immunized with MOG_{35-55}.

(A) Rag2$^{-/-}$ mice (n = 8) receiving Sema7A$^{-/-}$ T cells became moribund and died rapidly within 6–9 days following immunization, while mice receiving Sema7A$^{+/+}$ T cells (n = 8) remained healthy.

(B) Immunofluorescent detection of Sema7A$^{-/-}$ or Sema7A$^{+/+}$ CD4 (red, left) and detection of CD11b (red, right) in brain sections of TCR$\alpha^{-/-}$ mice. Results are representative of three experiments.

(C–E) Typical profiles of splenocytes from Rag2$^{-/-}$ recipients 6 days postimmunization. (C) Dilution of CFSE label in transferred CD4 T cells. (D) Expansion of CD T cells is greatly enhanced in Sema7A$^{-/-}$ cell recipients (n = 4/group ± SD). (E) Activated CD4 T cells with high CD25 surface staining are more numerous in spleens of Sema7A$^{-/-}$ CD4 than Sema7A$^{+/+}$ CD4 recipients.

of unstimulated cells (Figure 6B). Similar results were observed in CD8 T cells (data not shown).

As sustained surface expression of TCR is known to correlate with increased calcium signaling, we compared calcium mobilization upon anti-CD3 crosslinking in Sema7A$^{+/+}$ and Sema7A$^{-/-}$ CD4 T cells. We found that the magnitude of Ca^{2+} mobilization in Sema7A-deficient T cells was higher and decayed at a slower rate than in the control wt cells (Figure 6C).

TCR activation leads to rapid phosphorylation of ZAP-70 and activation of the ERK-MAP kinase pathway (Qian and Weiss, 1997). The comparison of tyrosine phosphorylation patterns between wt and Sema7A$^{-/-}$ T cells revealed no global differences in major phosphorylated bands, which include Zap70 and components of the TCR complex (data not shown). One of the downstream targets of TCR activation is glycogen synthase kinase 3 (GSK-3), which negatively regulates antigen-specific T cell proliferation by acting on nuclear factor of activated T cells (NF-AT) (Ohteki et al., 2000). In response to TCR signal, GSK-3 is inactivated by phosphorylation, which releases the inhibitory effect on T cell activation, while increased inhibition of GSK-3 leads to hyperproliferation of T cells (Ohteki et al., 2000). We observed a modestly increased inhibitory phosphorylation of GSK-3α/β on Ser21/9 in response to TCR stimulation in Sema7A$^{-/-}$ T cells, compared to wt T cells (Figures 6D and 6E). The S6 ribosomal kinase (S6K) is required for cell growth and G1 cell-cycle progression and is a target of TCR-induced PDK1 kinase activity (Lee et al., 2005; Pullen et al., 1998). We found that in Sema7A$^{-/-}$, T cells the phosphorylation of S6K is modestly increased in strength (Figure 6E) and duration (Figure 6D), in comparison to the wt T cells. TCR-induced ERK activation was similar in Sema7A$^{-/-}$ and wt T cells (Figures 6D and 6E).

Activation of T cells through the T cell receptor (TCR) triggers β1-integrin signaling and induces phosphorylation of focal adhesion kinase (FAK) (Iwata et al., 2000). Considering the evidence of Sema7A in β1-integrin function in neurons (Pasterkamp et al., 2003), we analyzed the FAK activation in Sema7A-deficient cells. We activated T cells with a combination of anti-CD3/CD28 antibodies. Within 10 min, the wt T cells exhibited a robust phosphorylation of FAK at Y925, which was diminished in the Sema7A$^{-/-}$ T cells (Figure 6F). We observed similar FAK phosphorylation defect in Sema7A$^{-/-}$ macrophages activated with collagen IV or with IGF-I (Figure S6A). TCR-mediated phosphorylation of FAK at Y925 creates a binding site for the adaptor proteins Gads/Grb2 which could negatively regulate T cell signaling by bringing inhibitory regulatory molecules into the proximity of the TCR complex (Schlaepfer and Hunter, 1996).

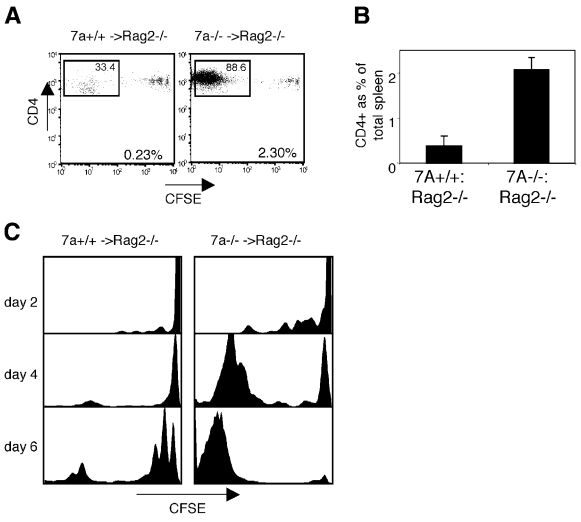


Figure 5. Homeostatic Proliferation of CD4 T Cells

(A) Increased homeostatic proliferation among Sema7A$^{-/-}$ CD4 cells compared to Sema7A$^{+/+}$ cells in lymphopenic host.

(B) Mean of CD4 T cells determined by surface antibody staining and FACS analysis (n = 4/group ± SD).

(C) Time course of homeostatic proliferation of transferred naive CD4 T cells labeled with CFSE and followed 2, 4, and 6 days after transfer. Shown are CFSE-dilution profiles of gated CD4 CD3 cells.

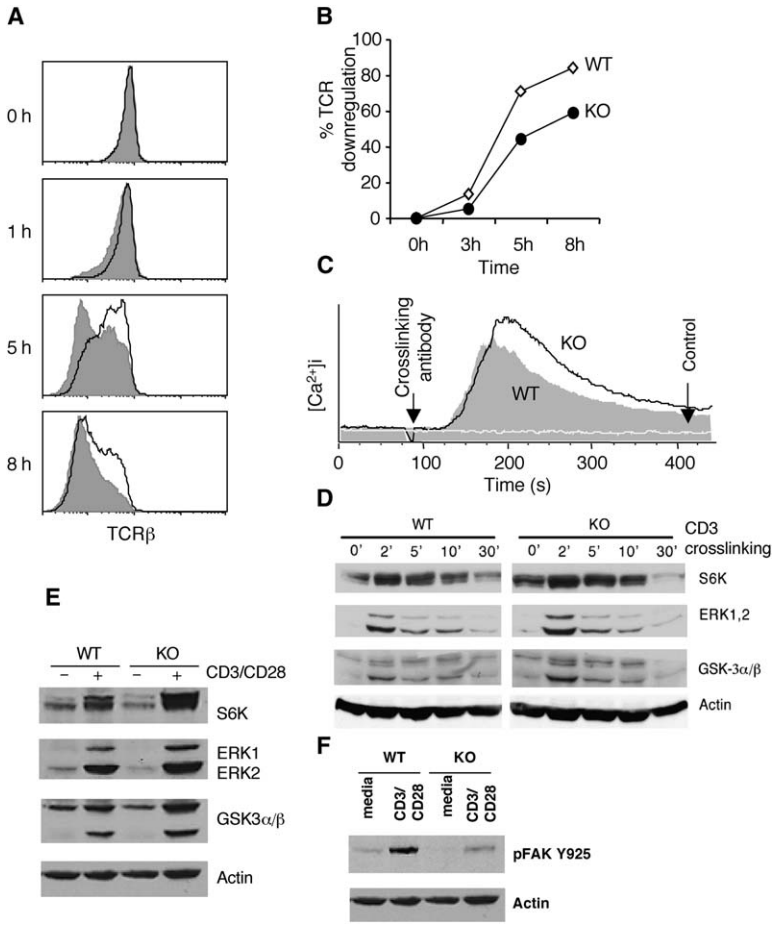


Figure 6. Analysis of TCR Signaling in Wt and KO CD4 T Cells

(A) Anti-CD3 stimulation induced TCR surface downregulation. T cells were stimulated with plate bound CD3 for indicated time, and surface TCR retention was determined by flow cytometry. Shown is TCRβ surface staining of gated CD4-positive population (filled histograms, wt; open histograms, Sema7A$^{-/-}$).

(B) Quantification of anti-CD3 induced TCR downregulation.

(C) Enhanced calcium mobilization in Sema7A$^{-/-}$ CD4 T cells. Histograms represent calcium flux measured by flow cytometry. Control: addition of secondary antibody alone.

(D and E) Lysates of naive CD4^{+} Sema7A$^{+/+}$ or Sema7A$^{-/-}$ T cells (D) coated with anti-CD3 antibody on ice and incubated with crosslinking antibody for indicated times, or CD4^{+} Sema7A$^{+/+}$ or Sema7A$^{-/-}$ effector T cells (E) activated for 10 min with anti-CD3/anti-CD28 antibodies were analyzed for kinases phosphorylation by Western blotting.

(F) Western blot of OTII CD4^{+} Sema7A$^{+/+}$ or Sema7A$^{-/-}$ effector T cells activated for 10 min with anti-CD3/CD28 antibodies shows diminished FAK phosphorylation. Results representative of three independent experiments.

Thus, with the exception of TCR downmodulation, Ca^{2+} mobilization, and FAK phosphorylation, Sema7A$^{-/-}$ T cell did not exhibit any dramatic enhancements in signaling events when stimulated with plate bound anti-CD3/CD28 antibodies in the absence of APCs. Furthermore, stimulation with anti-CD3, in contrast to the APC-induced hyperproliferation of Sema7A$^{-/-}$ T cells (Figure 1), led to a diminished Sema7A$^{-/-}$ T cells proliferation compared to wt T cells (Figure S6B). Activation of T cells with APC-independent stimuli such as anti-CD3 antibody crosslinking delivers a strong and nonspecific signal to the T cell and bypasses a number of signals that are otherwise delivered by an APC. Therefore, it is likely that while anti-CD3 activation allows visualization T cell signaling in the absence of contaminating APCs, the actual differences in signaling between wt and Sema7A$^{-/-}$ T cells could only be revealed when T cells are activated by professional APCs.

Sema7A$^{-/-}$ Functions in T Cell Activation by APCs

The hyperproliferation of Sema7A$^{-/-}$ T cells stimulated with DCs, but not with anti-CD3/CD28 antibodies in the absence of APCs, suggested that interaction of Sema7A with a receptor present on APC might be necessary to execute the inhibitory function of Sema7A in the T cell. To test this hypothesis, we activated Sema7A$^{+/+}$ and Sema7A$^{-/-}$ T cells with DCs pulsed with superantigen, SEA (staphylococcal enterotoxin A). This antigen-nonspecific activation resulted in hyperproliferation of Sema7A$^{-/-}$ T cells compared to wt cells (Figure 7A), similarly to the antigen-specific stimuli (Figure 1). This result implies an interaction of Sema7A with a receptor on DCs. Fixed DCs pulsed with SEA similarly resulted in an increased proliferation of Sema7A$^{-/-}$ T cells, indicating that Sema7A$^{-/-}$ T cells are not simply more responsive to other activating signals, such as cytokines, produced by DCs.

Sema7A may affect the TCR complex through a protein-protein interaction on the cell surface, and that interaction may be stabilized by the presence of a Sema7A receptor on the APC. Consistent with this prediction, we noticed that while staining of the TCRβ on the cell surface is identical in Sema7A$^{-/-}$ and Sema7A$^{+/+}$ cells (Figure 6A, top panel), staining of CD3 is decreased on Sema7A$^{-/-}$ CD4 T cells (Figure 7B). As the levels of TCR and CD3 are equal on the plasma membrane (Call and Wucherpfennig, 2005), this result suggests that CD3 in Sema7A$^{-/-}$ T cells is not fully accessible to the antibody, either because of a conformational change or obstruction of the surface part of CD3 by another molecule. Thus, Sema7A appears to directly affect the components of the TCR complex.

These results lead us to propose a tentative mechanism of Sema7A function in CD4 T cells. As shown in Figure 7C, left panel, Sema7A normally present on the surface of a T cell interacts with the components of the TCR/CD3 complex during T cell activation by an APC. A putative receptor present on APC (presumably

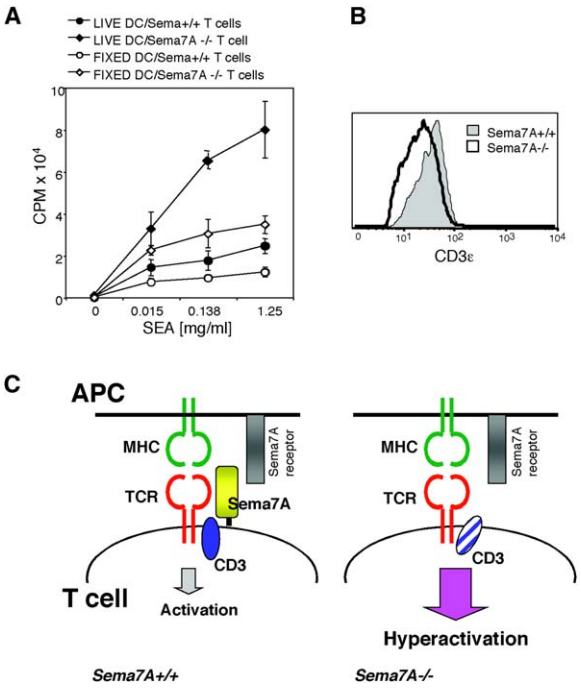


Figure 7. Sema7A Regulates T Cell Activation by APCs

(A) Sema7A$^{-/-}$ T cells activated with live of fixed DCs pulsed with staphylococcal enterotoxin A (SEA) show increased proliferation compared to wt T cells. T cell proliferative responses are expressed as the mean ± SE of triplicate cultures.

(B) Surface staining of CD3ε is decreased in Sema7A$^{-/-}$ CD4 T cells compared to Sema7A$^{+/+}$ cells.

(C) Model of Sema7A function in T cell signaling. Sema7A interacts with the components of TCR complex and with a putative receptor on APCs. This interaction stabilizes TCR/CD3 complex and promotes inhibitory signals that limit T cell proliferation. In the absence of Sema7A, the TCR/CD3 complex is destabilized, and upon activation by an APC, negative regulatory signals are impaired, leading to overt activation and T cell hyperproliferation.

β1-integrin) engages Sema7A, and that interaction negatively regulates the TCR. In the absence of Sema7A, this negative regulatory mechanism is lost, resulting in enhanced activation and hyperproliferation of CD4 T cells (Figure 7C, right panel).

Discussion

In this report, we describe a crucial function of a neuronal guidance factor, Semaphorin 7A, in the negative regulation of T lymphocyte function. We find that Sema7A-deficient T cells are hyperactive in response to activation in vivo and in vitro. This phenotype is particularly accentuated in wild-type lymphopenic hosts receiving small numbers of Sema7A-deficient T cells followed by immunization with self-antigen, which leads to a massive, unrestrained T cell expansion and a subsequent death of the host animal. This hyperactive phenotype correlates well with our finding that Sema7A knockout mice undergo a markedly different and more severe course of EAE compared to their Sema7A-sufficient littermates. The Sema7A-deficient animals are prone to die near the onset of clinical EAE, and that correlates with the increased infiltration of CNS by lymphocytes, which we observed in the moribund Sema7A knockout animals. We did not find any obvious neuron-specific role for Sema7A in neuronal survival and protection in the EAE model. We also provide evidence of an enhanced T cell-mediated delayed-type-hypersensitivity reaction in Sema7A-deficient mice, and this observation is in agreement with the general hyperactive phenotype of Sema7A$^{-/-}$ T cells. The hyperactivity of Sema7A$^{-/-}$ T cells is not dependent on antigen stimulation because the enhanced T cell proliferation is evident even in homeostatically proliferating T cells from Sema7A$^{-/-}$ mice. Collectively, these results demonstrate that Sema7A negatively regulates T cell activation and functions in a T cell-specific manner. Sema7A deficiency results in an enhanced T cell proliferation triggered by either antigen or lymphopenia. However, Sema7A-deficient mice do not develop any obvious autoimmune disorders, and T cell hyperactivity is most strongly pronounced under conditions of experimentally induced autoimmunity or lymphopenia, pointing to a specific, rather than general, inhibitory role of Sema7A.

Based on the T cell transfer experiments, we conclude that the mechanism of Sema7A activity is largely T cell intrinsic. Engagement of T cell receptors by an antigen leads to activation, cell proliferation, differentiation, and effector functions. It has been proposed that the initiation and propagation of the signaling events taking place in immune cells occur in specialized membrane regions, lipid rafts, where the lymphocyte receptors localize (Magee et al., 2002). The GPI (glycosylphosphatidylinositol)-linked proteins localize to lipid rafts, and many of them were shown to play important roles in T cell activation (Hwa, 2001; Loertscher and Lavery, 2002; Magee et al., 2002). We propose that similarly to several other T cell-specific GPI-linked proteins, Sema7A may interact in *cis* and negatively regulate the TCR complex within lipid rafts. Furthermore, our data indicates that Sema7A may somehow control the functional conformation of the TCR complex.

While many different biochemical pathways regulate T cell activation, TCR downmodulation and internalization are important means of negative regulation. Previous work utilizing E3-ligase Cbl knockout animals showed a critical role of TCR downregulation in T cell responsiveness, as in the T cells from these mice the abnormal retention of T cell receptor on the cell surface causes sustained TCR signaling (Naramura et al., 2002). In addition, Cbl-b knockout T cells show a hyperproliferative phenotype and develop autoimmunity, indicating that alterations in TCR downmodulation and negative regulation can lead to detrimental effects in the host (Bachmaier et al., 2000; Chiang et al., 2000; Krawczyk et al., 2000). We find that TCR downregulation upon stimulation is defective in Sema7A$^{-/-}$ T cells. The surface retention of TCR correlates with an enhanced and sustained calcium mobilization.

TCR-mediated cell signaling events are tightly regulated, as even small alterations in the TCR sensitivity can lead to changes in T cell activation and proliferation (Qian and Weiss, 1997). We found that CD4 T cells lacking Sema7A show slightly enhanced activation of S6K and calcium influx, as well as increased inhibitory phosphorylation of GSK-3. Increased inhibition of GSK-3 has been linked with T cell hyperproliferation (Ohteki et al., 2000). We also demonstrate a defect in activation of

FAK, which is reportedly involved in recruitment of negative regulators to the proximity of TCR (Schlaepfer and Hunter, 1996), although the functional significance of defective FAK activation is currently unclear. Interestingly, hyperresponsiveness of Sema7A-deficient T cells could only be observed upon T cell stimulation by professional APCs, suggesting a contribution of DC-derived signal to Sema7A-mediated negative regulation of T cell activation.

With the use of superantigen SEA, we find that an antigen-independent but APC-dependent activation of Sema7A-deficient T cells leads to their hyperproliferation and that increased response is preserved if fixed DCs are used as APCs. This suggests that part of the normal activation of T cells by APCs is the inhibitory signaling that limits T cell proliferation. It is likely that Sema7A interacts with a putative receptor expressed on DCs and that this interaction may be required to promote inhibitory function of Sema7A in TCR signaling.

Our data provide evidence for Sema7A function in TCR signaling that could involve either a direct association of Sema7A/TCR or possibly another molecule serving as the linker. The diminished antibody accessibility of surface CD3 in Sema7A$^{-/-}$ T cells indicates that the absence of Sema7A has a direct effect on the TCR complex composition or conformation, and that may contribute to the defects of T cell signaling seen in Sema7A-deficient T cells. The nature of the Sema7A counter structure remains puzzling. Recent report of the Plexin C1-deficient animals demonstrated only a small defect in T cell function in these mice and completely normal function of APCs (Walzer et al., 2005). While it remains possible that Plexin C1 plays some role in Sema7A signaling in T cells, it is likely that another receptor, possibly an integrin, plays a more relevant role. Our observations indicate that such receptor may function in *trans* with Sema7A and may be expressed on the DCs.

Based on the available evidence, we propose a model that predicts that Sema7A exists in a complex with the TCR and/or CD3 on the T cell surface. During T cell activation, Sema7A engages a receptor on the APC. This engagement may serve to change the conformation of Sema7A to make it active, or alternatively it may serve to position Sema7A within the T cell: APC synapse. Activity of Sema7A manifests in limiting T cell proliferation by promoting induction of the inhibitory signaling mechanisms. In the absence of Sema7A, TCR/CD3 signaling and complex assembly is impaired. This impairment may in part stem from the altered CD3 activity, defective TCR internalization, and altered intracellular signaling. In the absence of Sema7A, this negative regulatory mechanism is lost, resulting in an abnormally strong activation and hyperproliferation of the T cells. Conversely, providing additional soluble Sema7A inhibits T cell signaling and decreases proliferation.

Previous studies have demonstrated immunological function of several members of Semaphorin family, including Sema4A, Sema4D, and PlexinA1 (Bismuth and Boumsell, 2002; Kumanogoh and Kikutani, 2003; Wong et al., 2003). In each of these cases, the mechanism of Semaphorin or Semaphorin receptor function in the immune system is different. The present study, together with previous reports (Holmes et al., 2002; Mine et al., 2000), demonstrates that Sema7A also has an important function in the immune system, in addition to its function in the nervous system, and thus adds to a growing number of signaling pathways shared by the two systems (Trautmann and Vivier, 2001).

Experimental Procedures

Mice

Generation of Sema7A$^{-/-}$ mice was described previously (Pasterkamp et al., 2003). Male and female mice 4–6 weeks old were used for analyses. C57BL/6 TCR$\alpha^{-/-}$ and C57BL/6 RAG2$^{-/-}$ mice were purchased from the Jackson laboratory (Bar Harbor, ME) and Taconic Farms (Germantown, NY). Animals were housed in a pathogen-free facility at Yale University. All procedures involving mice were approved by Yale University Institutional and Animal Care and Use Committee.

Reagents and Antibodies

LPS, OVA, oxazolone, and staphlococcal enterotoxin A were from Sigma (St. Louis, MO). MOG_{35-55} and OTII OVA peptide were synthesized and purified by Keck Facility (Yale University, CT). APC anti-CD4, phycoerythrin (PE) anti-CD25, and rat anti-CD4 (L3T4) were all from BD Biosciences (San Diego, CA). Anti-rat IgG-PE was from Jackson ImmunoResearch Labs (West Grove, PA). Goat anti-rat Alexa Fluor 594 was from Molecular Probes (Eugene, OR). Anti-CD4, anti-CD11c, and anti-CD19 microbeads were from Miltenyi Biotec (Auburn, CA). Mouse cells were cultured in complete RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate (all from Sigma). Recombinant Sema7A-FC was prepared as follows: Sema7A with a deletion of C-terminal 25 amino acids was cloned into a pcDNA3 vector upstreem and in frame with a human Fc fragment, expressed in 293T cells, and purified from supernatant with protein A column (Amersham) according to manufacturer's instructions. The Fc fragment was expressed in pSecTag vector and purified by the same method. Recombinant proteins were dialyzed extensively against PBS and purity was assayed by UV absorption measurements.

EAE Induction and Scoring

For EAE induction, MOG_{35-55} (3 μg/ml in PBS) was mixed with equal volume of complete Freund's adjuvant (CFA, from Sigma), and 50 μl was injected subcutaneously in each flank of Sema7A$^{-/-}$ or Sema7A$^{+/+}$ littermates. Pertussis toxin (Ptx, 0.2 μg) (Biological Laboratories, Inc., Campbell, CA) was given intravenously (i.v.) at the time of immunization and again 2 days later. Mice were scored daily for clinical signs of disease, and a numerical score was assigned based on the severity of the disease symptoms: 0, no disease; 1, limp tail; 2, weak tail and partial hind limb paralysis; 3, total hind limb paralysis; 4, both hind limb and fore limb paralysis; 5, death. Mice with a score of 4 were euthanized. Remission was defined by a decrease in the score of at least one point for two consecutive days. EAE was considered remitting when at least one remission occurred within the first 26 days.

Adoptive Transfer

Pooled spleen and lymph node cells from Sema7A$^{-/-}$ or wild-type littermates were depleted with TIB 146, TIB 164, Y3JP, and TIB 128 hybridoma supernatants and rabbit complement (Cedarlane Lab). After depletion, cells were washed in PBS/1% fetal calf serum twice, stained with anti-CD4-APC, and sorted on FACSVantage. 1×10^6 CD4 cells were labeled with 5 μM Carboxy-fluorescein diacetate, succinimidyl ester (CFSE) for 7 min, washed in sterile PBS three times, resuspended in a final volume of 200 μl PBS, and administered intravenously to naive C57BL/6 TCR$\alpha^{-/-}$ or C57BL/6 RAG2$^{-/-}$ recipient mice, and EAE was induced 24 hr after transfer. For homeostatic proliferation studies, identical protocol was used, except the immunization step was omitted.

Staining and Flow Cytometry

Cells were stained with relevant antibodies for 30 min on ice, washed, and analyzed with a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software.

Immunization

For antigen-specific T cell responses, mice were immunized in hind foot pads with 100 μg/mouse of OVA (Sigma) and 50 μg/mouse of LPS emulsified in Incomplete Freund's adjuvant (IFA, from Sigma). Draining lymph nodes were collected 6 days postimmunization.

Cell Purification

Spleen and lymph nodes were harvested from 6–12 week old mice. Cell suspensions were incubated with anti-CD4 microbeads followed by purification with the AutoMACS sorter (Miltenyi Biotec). In all experiments, more than 95% of purified cells were positive for CD4.

T Cell Proliferation Assay

Purified CD4 T cells (1×10^5) from draining lymph nodes were cultured in flat bottom 96-well plates with spleen cells (3×10^5, 1500 rads irradiated) or bone marrow DCs (2×10^4) live or fixed, as indicated, and titrating doses of antigen for 72–84 hr. Proliferation of T cells was determined by incorporation of [^{3}H]thymidine for the last 12–16 hr of culture.

ELISA

Serum samples were collected by eye bleeding from animals. Paired antibodies for cytokine specific enzyme linked immunosorbent assays were from Pharmingen.

Immunohistochemistry and Immunofluorescence

Wild-type and Sema7A$^{-/-}$ animals were anesthetized, perfused with ice-cold PBS, and fixed with 4% paraformaldehyde in PBS. Brains and spinal cords were removed and frozen or paraffin-embedded 4–5 μm sections were prepared. Tissues were stained with anti-CD3 antibody, followed by histochemical development with DAB. For immunofluorescence, tissues were stained with anti-CD4 or anti-CD11b (BD Biosciences) and secondary anti-rat Alexa Fluor 594. Results representative of at least three independent experiments.

Histopathology

Slices (1 mm) of glutaraldehyde-perfused spinal cord (L5, L6, and S1) were postfixed in 1% osmium tetroxide/Millonig's buffer on ice for 90 min, dehydrated in ethyl alcohol (70%, 90%, 95%, and 100%), cleared in propylene oxide, and embedded in Epon. 1 μm epoxy sections were stained with toluidine blue for light microscopy. Sections were scored by an investigator blinded to the code, on a scale of 0 to 4 for cell infiltration, de- and remyelination, and Wallerian degeneration. Representative lesions were examined from matching levels of spinal cord in animals from both groups (Sema7A$^{+/+}$ and Sema7A$^{-/-}$) with comparable disease courses and clinical scores.

Calcium Mobilization

Purified CD4 T cells were resuspended in RPMI/10% FCS and loaded with 3 μM Indo-1 (AnaSpec, San Jose, CA) for 30 min at 37ºC. Cells were then washed with ice-cold PBS and surface stained with anti-CD3 for 30 min at 4ºC, followed by ice-cold PBS wash. Immediately prior to cross linking with secondary antibody, each sample was warmed up to 37ºC for 10 min, and calcium flux was measured with a LSRII flow cytometer. Data were analyzed with FlowJo software.

TCR Downregulation Measurement and Analysis

Single-cell suspensions of splenic cells were incubated for indicated times with 2 μg/ml plate bound anti-CD3ε at 37ºC. Collected cells were surface stained with FITC anti-TCRβ (H57-597), PE anti-CD4 or anti-CD8, fixed, and analyzed on FACS Calibur flow cytometer. The percentage of TCR downregulation for each gated CD4 or CD8 population was determined based on MFI of TCRβ staining on stimulated versus unstimulated cells. To calculate the percent of TCR downregulation from the cell surface, the following formula was used: (U, unstimulated; S, stimulated)

$$\%\text{TCR downregulation} = ([\text{MFI } U_{\text{time } 0} - \text{MFI } S_{\text{time } t}]/\text{MFI } U_{\text{time } 0}] \times 100$$

Western Blotting

Naive purified CD4 T cells were incubated on ice with anti-CD3 (10 μg/ml) for 30 min, washed with ice-cold PBS, and treated with secondary antibody for indicated times at 37ºC. Purified CD4 OTII transgenic T cells (wt or Sema7A$^{-/-}$) were cultured with irradiated wild-type spleen cells and OVA peptide (1 μg/ml) for 4 days. Cells were then purified, washed three times in PBS, and resuspended in RPMI medium without serum for 6 hr and activated with anti-CD3/anti-CD28 (at 10 μg/ml) for 10 min at 37ºC. 2×10^6 cells/sample were lysed immediately following activation; total protein (100 μg) was resolved on 10% SDS-PAGE gels. Blotting was performed with phospho-S6 kinase T389, phospho-ERK T202/Y204, or phospho-GSK3α/β S21/9 (all from Cell Signaling Technology), or monoclonal anti-β-actin (Sigma). Bands were visualized with the ECL System (all from Amersham).

Statistical Analysis

Analysis of statistical significance for indicated data sets was performed with Prism4 Graphpad software.

Supplemental Data

The Supplemental Data include six figures and are available at http://www.immunity.com/cgi/content/full/24/5/591/DC1/.

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